Zinc Transporters in Yeast

An Honors Thesis (HONRS 499)

By

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May 23, 2003

Abstract

The yeast *Saccharomyces cerevisiae* has several transporters for zinc regulation. Because zinc is an essential trace metal for proper metabolism, these transporters work to maintain a biologically sound intracellular environment for the yeast.

Each transporter has its own requirements for activation. Some transporters regulate the uptake of zinc through the plasma membrane, while others regulate the concentration of zinc through the vacuolar membrane. When the intracellular concentration of zinc falls below vital level, the transporters on the plasma membrane begin to uptake zinc from the surroundings, or perhaps if the yeast vacuole has an excess storage of zinc, vacuolar transporters may release zinc into the cell. Inversely, if the zinc concentration reaches a toxic level, the cell may activate the vacuolar transporter to store the surplus inside of the vacuole, as to not harm the cell.

The proposed functions of these transporters and their activation are examined using Fluorine NMR. A chelating reagent is added to the zinc-treated yeast to produce a signal for the NMR, which can aid in determining if the yeast is uptaking the metal and how much.

Acknowledgements

- I owe a great deal of gratitude to my advisor, Dr. Scott Pattison for teaching me so much in such a little timeframe. He patiently withstood all of my mistakes and endless questions. Thank you for your willingness to help me.

- Dr. Karlett Parra-Belky was essential in my preparation for the experiments. Without her, yeast would not grow properly and nothing would be sterile. Thank you for showing me how real biochemists work.

Introduction.

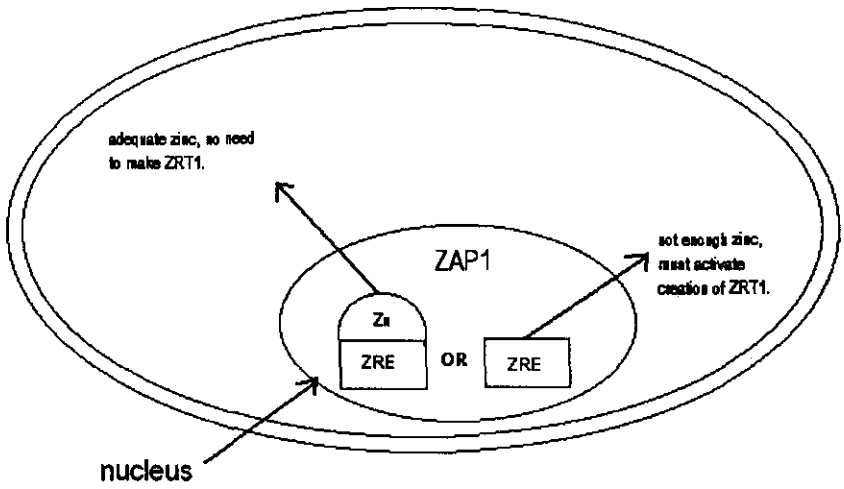
Zinc is an essential nutrient for life. It is not only required for gene transcription but is also used as a catalyst for many essential enzymes. Because it plays such a significant role in cellular metabolism, zinc deficiency can cause critical consequences.[1] For example, mammals with a moderate zinc deficiency might experience anemia, defective immune systems, developmental problems and improper embryonic development (teratogenesis). Despite the need for zinc, excess quantities can be just as detrimental. Too much zinc may promote neural changes similar to the neurofibrillary plaques seen in Alzheimer's. It may also suppress copper and calcium absorption. Copper is necessary for hemoglobin formation in red blood cells, enzymes and collagen while calcium is necessary for healthy bones and strong teeth. Because a delicate balance of zinc is needed, cells must maintain an adequate intracellular zinc level to meet cellular requirements while preventing metal ion overaccumulation. A number of intermembrane transporters in the cell help to regulate this critical concentration. This study examines the tightly controlled storage of zinc in an intracellular organelle called a vacuole.

The *Saccharomyces cerevisiae* yeast provides an excellent model system in which to study zinc uptake in a eukaryotic cell. Biochemical assays of zinc uptake in yeast indicate that the process is transporter-mediated. The presence of five zinc-specific regulators has been demonstrated. Two of these five are plasma membrane systems – one system having a high affinity for zinc, the other having a lower affinity. These systems are ZRT1 and ZRT2, respectively.

ZRT1 is a zinc regulated transporter located in the plasma membrane of a yeast cell. It is a protein that contains an amino acid sequence that is thought to be a metal-binding domain in which one histidine of a group of four is located approximately 30 amino acids from the carboxyl end may contribute to the metal binding. This histidine-rich domain is found in a large loop that is predicted to be located on the cytoplasmic surface of the membrane. On the extracellular surface of the membrane, the protein has carbohydrates attached to provide polarity. The molecular mass for ZRT1 is anywhere between 53 and 63 kDa (8.79×10^{-20} to 1.04×10^{-19} grams). Immunoblot tests have shown a few bands in the 45 kDa region with one band in the 31 kDa region. This separation is due to the carbohydrates attached to parts of the protein. Because the additional carbohydrates would account for a heavier molecular weight, the band in the region around 45 kDa would represent some of the protein with the carbohydrates still attached, while the band in the 31 kDa region would represent only protein fragments.[2]

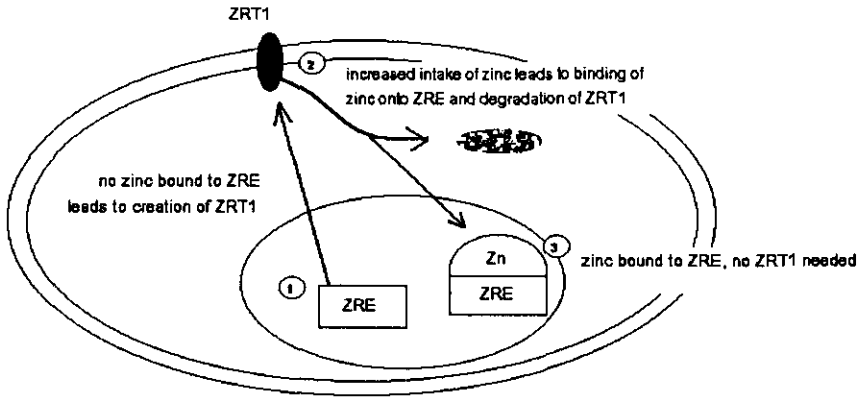
ZRT1 is regulated by intracellular cytoplasmic zinc pools, which, when experiencing a high zinc concentration (one higher than the steady-state level of $\sim 0.07 \times 10^{-15}$ moles/liter), can signal the inhibition of the production of the protein through the ZAP1 transcriptional activator. When the concentration of zinc drops below the vital level, and ZRT2 (a transport protein homologous to ZRT1) has already been activated, ZAP1 activates the production of a ZRT1 protein, which is created and then relocated to the plasma membrane so that it can once again uptake extracellular zinc, but at a faster rate than ZRT2. The zinc-responsive element (ZRE) senses the need for zinc and activates the production of ZRT1.

Figure 1. How the ZAP1 activator functions: if an adequate supply of zinc is on hand, the ZRE is inactive and there is no need to make ZRT1 (ZRT2 is enough). If zinc is nearing depletion, the ZRE is activated, which activates the production of ZRT1.



When the cell is experiencing a boost in zinc concentration, the excess zinc inhibits and therefore shuts off the ZAP1 activator (ZRE), which in turn stops the ZRT1 protein from transporting zinc into the cell, a process occurring at the transcriptional level. ZRT1 is then engulfed by the surrounding membrane and pinched off (a form of zinc-induced endocytosis) and then degraded, a process occurring on a post-translational level.[6] This process protects the cell from being exposed to high zinc levels and prevents zinc overload.

Figure 2. Process of the ZAP1 activator: first the ZRE triggers the production of ZRT1, which is used to take in zinc much faster; once the zinc in the cell is restored, the ZRE is shut off, making ZRT1 obsolete. The protein is eradicated.



ZRT2 can be thought of as the primary zinc transporter for the plasma membrane, although not at the maximum rate. When much zinc is needed, the cell activates the production of ZRT1, which is capable of uptaking large amounts of zinc so that the cell is able to restore its supply. ZRT2 can be seen as a high-affinity protein, but in comparison to ZRT1, it is low.[3] This concept of ZRT1 leads scientists to believe that ZRT1 is necessary for a high-affinity system (induced by zinc limitation) when ZRT2 is not fulfilling the cell's zinc needs. On the other hand, a lower-affinity system (also induced by zinc limitation) is regulated by ZRT2, when the cell has adequate zinc levels and no transport overdrive is needed.[4] ZRT1 and ZRT2 are both members of the ZIP family of metal ion transporters found in eukaryotic organisms. ZIP transporters are typically characterized by eight membrane-spanning helices that transport their particular metal ion

through using a proton motive force. Members of the ZIP family are found in animals, plants, yeast, and bacteria. The two yeast proteins, ZRT1 and ZRT2, have similar functions – both bring zinc in from extracellular surroundings for use in the cells, the activity of both is regulated on transcriptional and post-translational levels, but each perform under different circumstances. ZRT1 transports zinc with a ten-fold higher affinity than ZRT2.[4]

The remaining three of the five zinc-specific regulators of yeast include a transporter that exports zinc from the vacuole to the cytoplasm and two that store the cytoplasmic zinc into the vacuole. ZRT3 is a zinc transporter located in the vacuolar membrane of yeast. Little is known about its structure. It is a protein made up of a sequence of amino acids which (because it is known to be a member of the ZIP family of metal ion transporters) would contain eight membrane-spanning helices that transport their particular metal ion through the membrane using a proton motive force. While ZRT3 plays an analogous role to ZRT1 and ZRT2 by supplying zinc to the cytoplasm, it performs this function in the vacuolar membrane rather than the plasma membrane. ZRT3 is required to mobilize stored zinc inside of the vacuole during the transition from zinc-replete to zinc-deficient conditions. It plays a major role in controlling the transportation of vacuolar zinc stores. When the concentration of the zinc in the cytoplasm drops below vital levels, the ZAP1 transcriptional activator can also signal the production of ZRT3. The activity of the protein promotes zinc transport out of the vacuole and into the cytoplasm, where it becomes available for use.

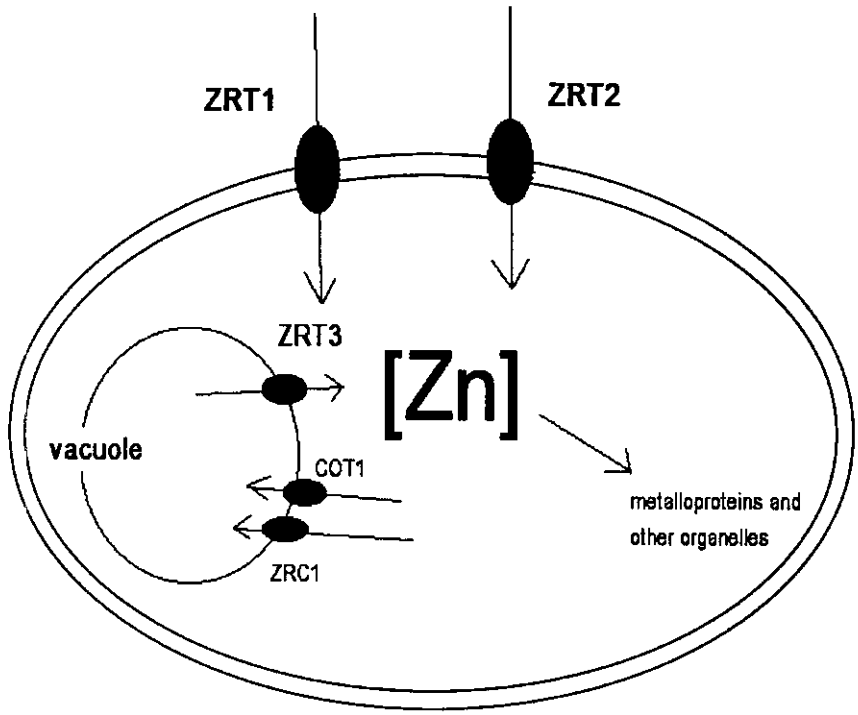
It is not known how the cell chooses which transporter to produce. Both ZRT1 and ZRT3 are created in low zinc situations, each transporting zinc into the cytoplasm. A speculative theory to explain such behavior may be that the ZAP1 activator can not only sense when the cytoplasmic zinc concentration is low, but also when the vacuolar concentration is low. If the cell has adequate zinc stored in the vacuole, perhaps ZAP1 activates ZRT3 rather than ZRT1. If the cell does not have adequate zinc stores, ZAP1 most likely activates ZRT1 to begin intake.

ZRC1 is a zinc transporter in the vacuolar membrane. It is a member of the CDF (Cation Diffusion Facilitator) family, whose structural characteristics include six transmembrane domains containing histidine-rich motifs that are predicted to be on the cytoplasmic surface of the membrane.[5] When ZRC1 is induced, the level of intracellular zinc decreases, meaning that ZRC1 transports zinc into the vacuole. The ZRC1 promoter has a ZRE (zinc-responsive element) and activation of ZRC1 is regulated in a ZAP1 fashion (a transcriptional factor critical in zinc-responsive genes). This means that the ZRC1 protein is created when it is needed (when there is excess zinc in the cytoplasm). The protein senses zinc availability in the cytoplasm, which might be through the histidine motifs, and transports zinc from the cytoplasm to the vacuole if zinc is abundant in the cytoplasm. On the other hand, if the cell is exposed to zinc-limited conditions, ZAP1 activates ZRT1, ZRT2 and ZRT3 to enhance the uptake of zinc from the extracellular surroundings (via ZRT1 and ZRT2) as well as from the vacuole (via ZRT3).

COT1, a transporter protein homologous to ZRC1, is also located in the vacuolar membrane. It too transports excess zinc from the cytoplasm into the vacuole. It is also a member of the CDF family, meaning it has six transmembrane domains containing histidine-rich motifs on the cytoplasmic surface. Both COT1 and ZRC1 transport cytoplasmic zinc into the vacuole for storage.[5]

The following figure represents the overall picture of how a yeast cell imports and exports zinc.

Figure 3. The big picture. ZRT1 and ZRT2 both take in zinc from extracellular surroundings, ZRT2 being the everyday transporter while ZRT1 appears when necessary. ZRC1 and COT1 both store excess zinc, and ZRT3 releases it when needed.



The theory for the proposed experiment involves the use of a chelating agent called BAPTA (1,2-Bis(2-amino-5-fluorophenoxy)ethane-N,N,N',N'-tetraacetic acid

tetrakis(acetoxymethyl) ester). BAPTA is a large molecule with metal-binding regions and two prominent fluorines. Because of these fluorines, a mix of BAPTA and yeast would give a signal in ^{19}F NMR. The signal is useful because it represents the BAPTA that has zinc bound to its metal-binding regions, and the fluorine signal would correlate to the amount of zinc bound. Mechanistically, BAPTA is introduced into the yeast and is able to flow freely in and out of the cells. However, once a metal ion is bound to BAPTA, the structural conformation changes as does the chemical reactivity, making it unable to leave the cell. The BAPTA-zinc complex is now trapped inside of the yeast cell, and a fluorine spectrum of the complex can be analyzed and interpreted.

Materials and Methods.

Yeast strains and culture conditions: Strains used were wild type (no mutations or deletions) grown in a standard culture media of YEPD (yeast extract, peptone, and dextrose). To prepare, 10 grams of yeast extract (Fisher Scientific Biochemicals, catalog number BP1422-500) and 20 grams of peptone (Fisher Scientific Biochemicals, catalog number BP 1420-500) are added to 960 mL deionized water to dissolve solids. This solution is autoclaved for 15 minutes on liquid cycle 2 (45 psi at 250° F). The dextrose solution is prepared by dissolving 300 grams of anhydrous dextrose (Fisher Scientific Biochemicals, catalog number BP350-1) into 300 mL deionized water and adding small volumes of additional water so that the final volume equals 600 mL (some of the initial water will be absorbed by the dextrose). Add 40 mL of this solution to the cooled YEP solution. Yeast grown in a petri dish are gently swabbed with a sterilized wooden stick and transferred to a sterile test tube holding a few milliliters of YEPD. This is incubated

for 2 hours at 37°C and 212 rpm in a New Brunswick Scientific Classic C25KC incubator shaker (serial number 001088951). After 2 hours, transfer entire contents of test tube to a sterile 250 mL flask and add 40 mL more of YEPD. Incubate again for 2 hours under same conditions mentioned above. After 2 hours, take a UV-Vis spectrum (Hewlett Packard, model number 8452A) at 600 nm to examine the optical density (OD). In order to have yeast functioning at a maximum, they must be harvested at a log growth phase – an OD reading of 6 correlates to this optimum growth phase. Because 1 OD is equal to 10^7 cells, we are looking to transfer roughly 6 x 10^7 cells. The following example calculations display the method in determining the amount of incubated yeast to add to a 1L flask of YEPD.

1:10 dilution of yeast in the cuvette gives reading of 0.02877 OD/mL

Because the flask is 10 times more concentrated: (0.02877)*(10) = 0.2877 OD/mL

0.2877 OD in 1 mL→ how many mL in 6 OD?

0.2877 OD/ 1mL = 6 OD/x mL

x = 6/0.2877 = 20.86 mL

This indicates that 20.86 mL are appropriate to add to the 1L flask of YEPD to give the desired OD after incubating overnight (minimum of 16 hours). When finished incubating, it is necessary to separate the cells from medium. Using a HEPES buffer and a centrifuge, the cells can be concentrated and ready for NMR use. The HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer is prepared by dissolving 5.96 grams HEPES and 2.92 grams NaCl into 450 mL pure water. HEPES is an organic buffer used in cell culture to maintain physiological pH in the presence or absence of a CO_2 incubator. Once all solids are dissolved, setup a pH meter and add concentrated

KOH dropwise until the pH reads 7.0. Add this mixture to a 500 mL volumetric flask and dilute as indicated.

Take cells out of incubator and transfer to several centrifuge tubes. Spin them down at 3000 rpm for 10 minutes. Discard the supernatant and wash the pellet with 25 mL HEPES buffer. Spin again for 10 minutes. Discard supernatant, wash again with another 25 mL buffer. Repeat this procedure 2 more times (for a total of 3 washings). After third spin, discard supernatant and combine "sludge" of cells into one container. Use less than 1 mL for an NMR tube and refrigerate the remainder. Throw out unused portion after 2 days.

Results and Discussion.

Several ^{19}F NMR readings of yeast and BAPTA gave similar results of a broad peak with excessive noise. The spectrum looks rough and non-specific because the material being examined is biological – made up of several macromolecules – where NMR is mostly used for analysis of simple compounds. The yeast is an atypical NMR specimen and so the results are expected to be less than neat. A representative spectrum of these readings entitled Spectrum 1 follows this report. The large peak represents the signal from the fluorines in BAPTA, and the integral area of the peak at -116 ppm represents the amount of zinc the fluorines neighbor. Although it is apparent that there is a definite signal, the broadness of the peak depicts a possible masking effect. Because of all the other macromolecules in the specimen and given the broad signal with a poor signal to noise ratio, the signal for zinc alone is almost impossible to identify. The

intensity is also less than desirable. Because this peak is not sharp and very broad, the results taken from it are rather inconclusive.

In addition to the broad spectrum, the question of whether the BAPTA-zinc signal was coming from inside or outside the cell was raised. The addition of zinc to the yeast provides for uptake but does not ensure it. Unlike BAPTA, zinc cannot be trapped inside of the yeast. Thus it can enter or leave at any moment. Also, there is no guarantee that all of the BAPTA has entered the cell. Because it is possible to have a significant amount of BAPTA-zinc complexes both inside and outside the cell, it was unknown whether the peak was a result of intracellular zinc or extracellular zinc. In order to ensure that the signal was from intracellular BAPTA-zinc complexes, an EDTA solution (0.0005 *M*) was added. EDTA, or ethylenediaminetetraacetic acid, is an excellent chelator that is able to bind any free-floating zinc outside of the cell, eliminating the possibility of an extracellular signal. Even if zinc is bound to BAPTA outside of the membrane, EDTA is strong enough to take and bind that zinc to itself. EDTA is too large to be transported across the plasma membrane, meaning it will only eliminate extracellular zinc because it is trapped on the outside. This ensures that the signal received is from intracellular BAPTA-zinc complexes alone. Spectrum 2 represents the signal after the addition of EDTA. The spectrum shows a sharpening of the original messy peak, as well as increased intensity. This is an indication of the EDTA serving its purpose – it is cleaning up any and all excess metal ions outside of the cell. It is understood now that the sharp, intense peak in Spectrum 2 represents only BAPTA-zinc complexes inside of the cell.

Future experiments might involve greater concentrations of zinc, BAPTA, and EDTA to perfect the zinc signal from the NMR. The addition of EDTA tests for

BAPTA-zinc complexes on the outside. A similar test would be to add other divalent metals, like Mn^{2+} or Fe^{2+}. This change would give BAPTA different binding options, changing the position of the BAPTA peak on the spectrum, and preventing the passage of BAPTA into the cell. This test, along with EDTA, would investigate the conditions for BAPTA and zinc on the inside of the cell only. For example, changing the glucose amounts or the pH of the cells may change how BAPTA is taken in. It is also possible that BAPTA is entering the cell but being exported by a multi-drug transporter before it can bind with zinc and become trapped. A multi-drug transporter rids the cell of foreign molecules seen as poisons in an attempt to safeguard the cell. To ensure that the BAPTA is not being exported before it can be trapped, the cells could be treated with a multi-drug transporter inhibitor to shut off the cell's defense to foreign molecules.

To test the functions of the many transporters previously discussed, yeast mutants would be used. For example: to determine if ZRC1 and COT1 effectively store zinc into the vacuole, the genes *zrc1* and *cot1* would be deleted through an induced mutation. The resulting yeast mutant would be subjected to the conditions previously mentioned of zinc, BAPTA and EDTA. The hypothesized result would be that the mutant would die of zinc toxicity as a result of having no place to store the excess zinc. If this did not happen, the role of ZRC1 and COT1 would have to be reexamined. Likewise, the other transporter functions could be examined closer using the same mutant techniques.

Spectrum 1

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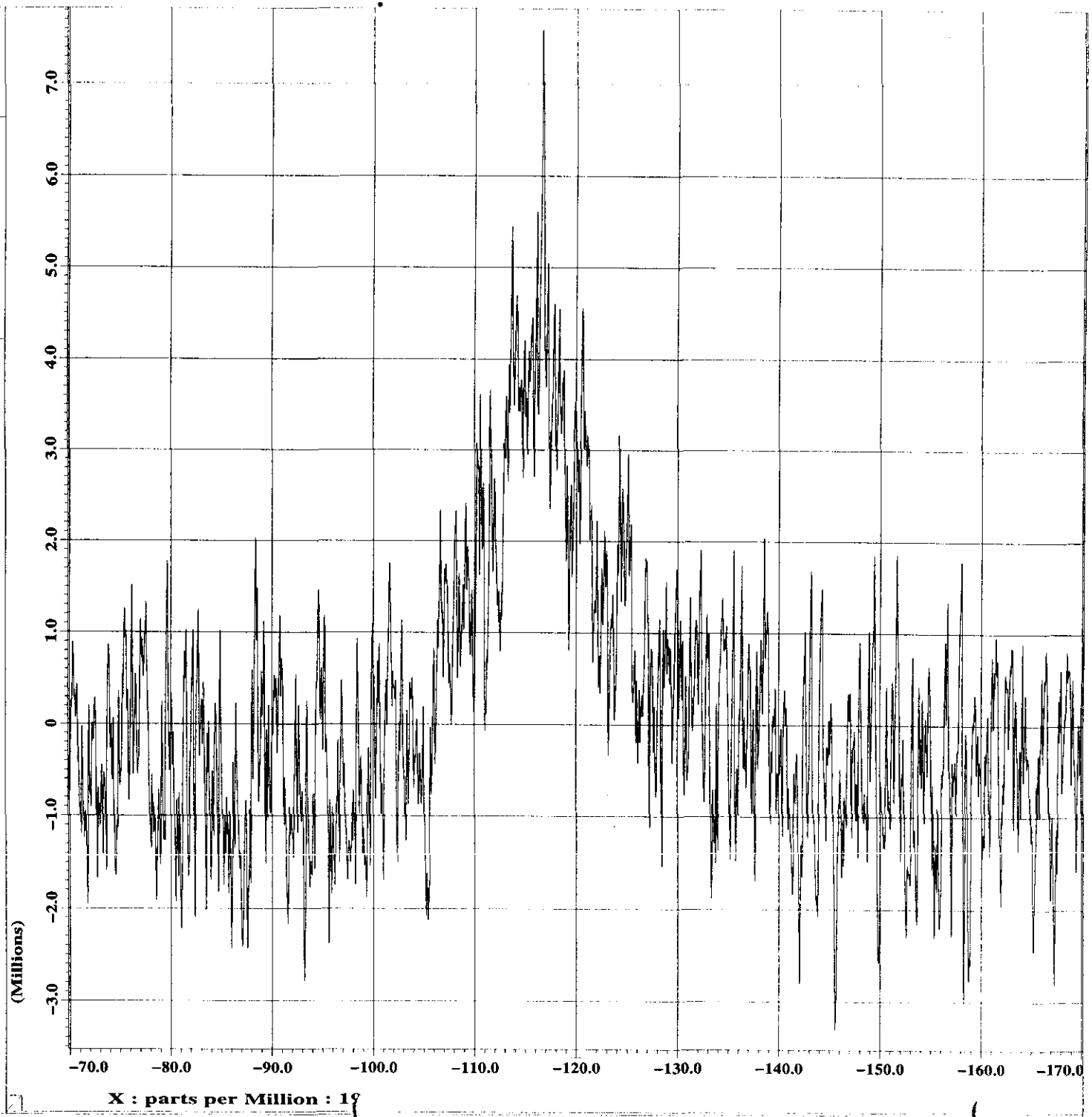

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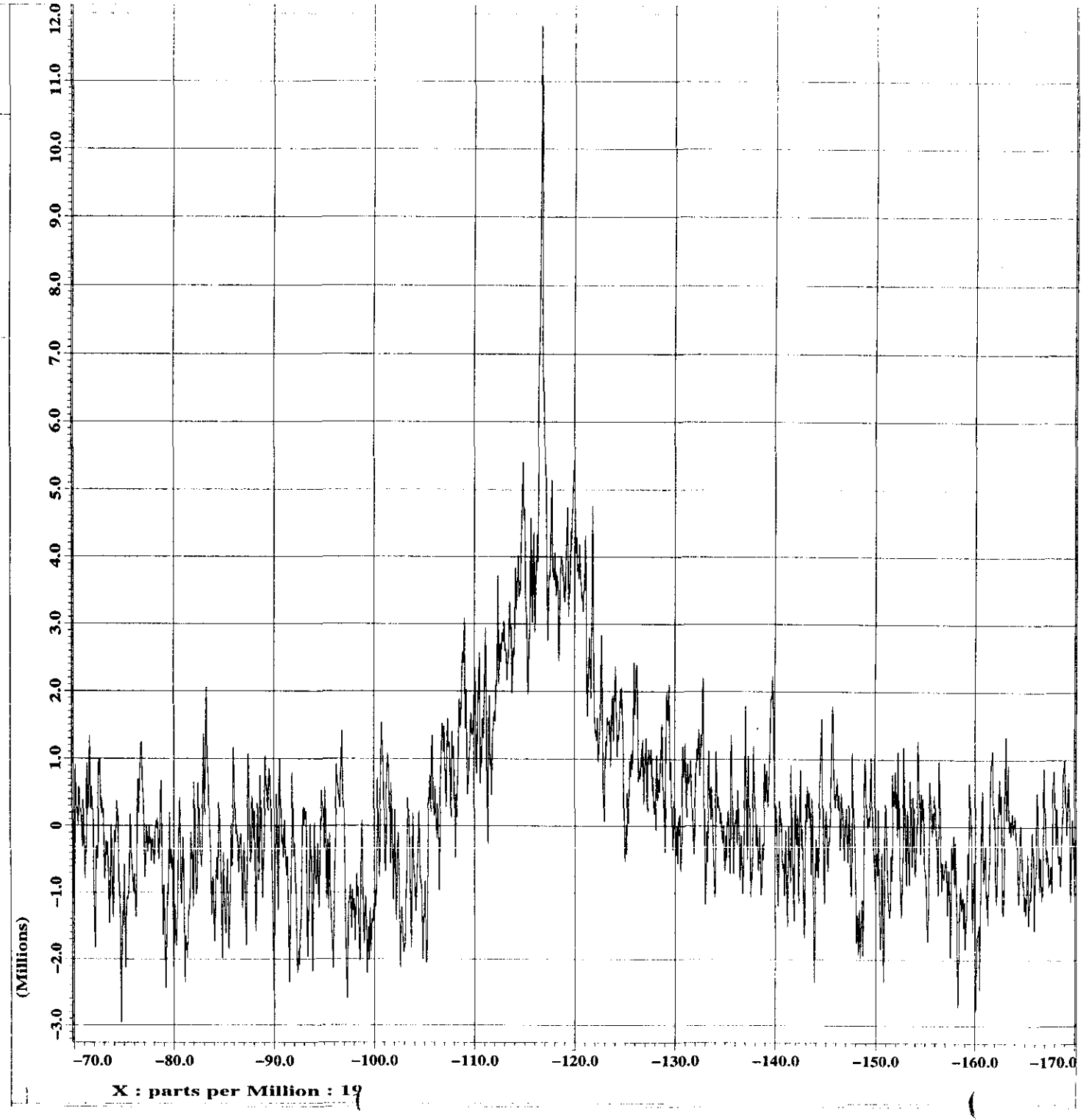

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